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Control of artefactual variation in reported inter-sample relatedness during clinical use of a *Mycobacterium tuberculosis* sequencing pipeline

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ABSTRACT

Contact tracing requires reliable identification of closely related bacterial isolates. When we noticed the reporting of artefactual variation between *M. tuberculosis* isolates during routine next generation sequencing of *Mycobacterium spp,* we investigated its basis in 2,018 consecutive *M. tuberculosis* isolates. In the routine process used, clinical samples were decontaminated and inoculated into broth cultures; from positive broth cultures DNA was extracted, sequenced, reads mapped, and consensus sequences determined. We investigated the process of consensus sequence determination, which selects the most common nucleotide at each position. Having determined the high-quality read depth and depth of minor variants across 8,006 *M. tuberculosis* genomic regions, we quantified the relationship between the minor variant depth and the amount of non-Mycobacterial bacterial DNA, which originates from commensal microbes killed during sample decontamination. In the presence of non-Mycobacterial bacterial DNA, we found significant increases in minor variant frequencies of more than 1.5 fold in 242 regions covering 5.1% of the *M. tuberculosis* genome. Included within these were four high variation regions strongly influenced by the amount of non-Mycobacterial bacterial DNA. Excluding these four regions from pairwise distance comparisons reduced biologically implausible variation from 5.2% to 0% in an independent validation set derived from 226 individuals. Thus, we have demonstrated an approach identifying critical genomic regions contributing to clinically relevant artefactual variation in bacterial similarity searches. The approach described monitors the outputs of the complex multi-step laboratory and bioinformatics process, allows periodic process adjustments, and will have application to quality control of routine bacterial genomics.

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INTRODUCTION

Identifying closely related bacterial isolates is required for clinical and epidemiological purposes (1-3). Most published approaches using short-read next generation sequencing (NGS) rely on mapping to a high-quality reference sequence followed by consensus base calling (1-8). A known problem with this approach concerns the existence in many bacterial genomes of 'hard-to-map' regions which are either repeated within the genome, or which contain regions of low sequence complexity. High-confidence mapping of short reads to such regions is difficult or impossible, and so determining the consensus sequence of these regions is difficult. One approach to managing this problem is to identify these regions bioinformatically prior to mapping by analysis of sequence complexity (9), or from repetitiveness within the genome (10). Base calls within these pre-specified regions are then ignored ('masking') when assessing relatedness of isolate sequences. A second complementary approach filters base calls based on read mapping confidence as reported by various mappers (11-14) in the form of Mapping Quality (MAQ) scores.

Mycobacterium tuberculosis is one of the most important pathogens of humans, with about 3 million cases of TB confirmed by culture globally each year (15). Recently, laboratory protocols have been described and deployed by Public Health England (4) in which the species and drug resistance of *Mycobacteria,* including *M. tuberculosis*, are identified by sequencing microbial DNA. Laboratory processing of clinical samples suspected of containing *Mycobacteria* involves decontamination using chemicals which kill non-Mycobacterial species before the samples are inoculated into broth culture (16). Mycobacterial Growth Indicator Tubes (MGIT) and associated tube monitoring equipment are a commercially available implementation of such a broth culture system.

In the process adopted by Public Health England, sequencing and bioinformatics analysis of DNA extracted from positive MGIT tubes allows determination of Mycobacterial species and drug resistance (17). This laboratory and bioinformatic process also allows the genetic distance between *M. tuberculosis* isolates to be estimated, using sequences derived by consensus base calling from mapped data. The organism co-evolved with human populations as they migrated, generating multiple lineages which differ from the ancestral sequence by hundreds or thousands of single nucleotide polymorphisms (18), as well as small indels, gene deletions and inversions (19). However, the evolutionary clock rate of the organism is slow at about 0.5 single nucleotide variants (SNV)/genome/annum (3, 5, 7) and small numbers of single nucleotide variants (SNV) are of clinical significance: studies based on retrospective collections of *M. tuberculosis,* grown on solid media prior to sequencing, have proposed thresholds of 5 SNVs as compatible with recent transmission (3, 5, 7). The bioinformatic processes used for relatedness estimation in the deployed pipeline were

also optimised using samples re-grown from frozen stocks on solid media (Lowenstein-Jensen slopes) (16).

Quality of complex processes deployed in medical laboratories is assured by adherence to quality standards, such as those laid out in ISO15189 (20). These standards require that the processes followed, and the environments in which they operate, comply with to patterns of work known to enhance the consistency and interpretability of the laboratory outputs. For example, in a drug testing laboratory a set of samples of known composition may be run through the analysers to confirm that particular commonly found substances which might potentially interfere with the assay (such as caffeine, paracetamol, and so on) have no impact on detection of the drug of interest. *M. tuberculosis* infection is commonly diagnosed from sputum samples, which contains a wide variety of organisms other than *Mycobacteria* (21). DNA from such organisms may contain sequences homologous with those present in *Mycobacteria*, for example in highly conserved core bacterial genes. As such, this non-Mycobacterial DNA has the potential to interfere with assays based on mapping of Mycobacterial reference genome mapping.

Here, we investigate the concept of interfering substances in the context of the detection of closely related *M. tuberculosis* isolates. In particular, we consider whether DNA of non-Mycobacterial origin might cause interference. In doing so, we describe a process which we call *adaptive masking.* This defines 'hard-to-map' regions existing in the context of the laboratory, sequencing and mapping processes being used, independently of predictions based on the reference sequence, and of filtering based on reported mapping quality. Our work was motivated in part by observations from analysis of prospective sequencing of *M. tuberculosis* sequences in England using a previously described bioinformatics pipeline (17). It appeared that high SNV distances were being reported between isolates with a strong epidemiological likelihood of having recently transmitted to each (e.g. isolates with unusual resistance profiles from individuals who were co-habiting): that is, false positive variation between isolates was being reported. We assess the impact of adaptive masking on addressing this problem, and discuss quality control of relatedness monitoring in the context of continuous process monitoring in accredited clinical laboratories.

METHODS

Isolation of DNA from Mycobacteria and Sequencing

Clinical specimens were decontaminated and inoculated into Mycobacterial Growth Indicator Tubes (MGIT) tubes (16). Positive samples were extracted (4). After DNA extraction, Illumina sequencing libraries were prepared using either 11 (early in the study) or 15 (later in the study) Mycobacterial DNA extracts, as previously described (4). All samples sent from patients for processing for *Mycobacteria* between 1/05/2016 and 30/05/2017 to a single reference laboratory were studied; the catchment of this laboratory is approximately 15 million people, or about one third of England.

Bioinformatic processing

Reads obtained from the MiSeq instrument were first examined for the presence of *Mycobacterium tuberculosis* using the Mykrobe tool which detects species-specific k-mers (22). Only samples identified as being derived from the *M. tuberculosis* complex by Mykrobe(22) were considered in this work. Additional read classification was performed with Kraken (23), which assigned reads to bespoke database constructed from (i) all bacterial genomes deposited in the NCBI RefSeq database as of January 2017 and (ii) Genome Reference Consortium Human genome build 38 (GRCh38) to allow detection of host DNA as described(24), but with k-mer reduction to 25 gigabases. We quantified reads mapped to *M. tuberculosis* (NCBI taxid: 77643), to non-Mycobacterial bacterial species, and to humans. After doing so, human reads were discarded.

Reads were mapped to the H37Rv v2 genome (Genbank NC_000962.2) using Stampy (14), as described (4). Samtools (25) was used to assess sequencing and mapping quality: high quality bases were considered to be those passing the –q30 and–Q30 30 filters (read quality and mapping quality all >30). Consensus sequence was called, requiring a minimum-read depth of 5, including at least one read on each strand. Where an alternative base represented more than 10% of read depth, the base was recorded as uncertain, as described (26).

The variant call format (VCF) file was parsed with custom python scripts, and the number of high quality bases (defined using the filters above) at each position determined. These frequencies were extracted, stored and indexed using SQLite using a python API constructed to allow extraction of mixture frequencies in arbitrary positions.

Modelling minor variant frequencies

We determined the most common (major) variant at each position. All other variants are considered minor variants. We define

n - the total sequencing depth at one base;

136 m - the depth of most common variant at one base;
137 m' - the depth of all variants other than most common, n-m (Suppl. Fig. S1).
138
139 We divided up the H37Rv genome based on the annotation in NC_000962.3, identifying 8007
140 regions R, comprising open reading frames and regions between open reading frames
141 (Supplementary Data S1).

142 For each of these regions j=1..8007, if the region has length l_j, the total number of minor variant
143 bases V_j across the i=1..l_j bases in the region is given by equation (1) and the total read depth D_j
144 across the gene by equation (2).

145 $$V_j = \sum_{i=1}^{l} m_l' \qquad (1)$$

146 $$D_j = \sum_{i=1}^{l} n_l \qquad (2)$$

147
148 In order to describe the relationship between non-Mycobacterial DNA quantifications and minor
149 allele frequency, we stratified the number of reads from each sample identified as being from
150 bacterial genera other than *Mycobacterium (b)* into four approximately equally sized strata:
151 $b < 1\%$,
152 $1\% \leq b < 5\%$,
153 $5\% \leq b < 20\%$,
154 $b \geq 20\%$.
155
156 We constructed separate Poisson regression models relating minor base counts (V) for each of the
157 8,007 regions (with log link and offset log(D)) to the non-Mycobacterial bacterial read categories b
158 (reference category < 1%), excluding any samples with zero high-quality depth in that region. We
159 applied Bonferroni correction to model outputs to control for multiple testing (α = 0.01/8007 =
160 1.2×10^{-6}).

161 ## Comparing impact of masking on pairwise comparisons

162 Based on analysis of model output (see Results), regions with higher minor variant counts than
163 expected were identified. These regions were excluded from pairwise comparisons performed using
164 the findNeighbour2 tool (27).

165 ## Availability of software and data

166 The samples studied have been submitted to NCBI with project number PRJNA451404. Software to
167 effect the process described, test data, links to the data used, and documentation is freely available
168 at http://github.com/davidhwyllie/adaptivemasking.

Impact of mapper

For a random 250 sample subset, we compared the impact of different mappers on minor variant frequencies across regions using the pipeline above. We compared mapping with Stampy 1.0.32(14), and Bowtie v 1.2.2 (28) with default parameters, and Bowtie2 v 2.3.4.1 with –very-sensitive and –very-fast preset parameters (29).

Ethical framework

Public health action taken as a result of notification and surveillance is one of the Public Health England's key roles as stated in the Health and Social Care Act 2012 and subsequent Government directives which provide the mandate and legislative basis to undertake necessary follow-up. Part of this follow-up is identification of epidemiological and molecular links between cases. This work is part of service development carried out under this framework, and as such explicit ethical approval is unnecessary.

RESULTS

Samples studied

The PHE National Mycobacteriology Reference Laboratory Midlands implemented a laboratory process in which specimens received are decontaminated, and inoculated in MGIT bottles, DNA extracts from positive MGIT bottles made, and their contents determined using Illumina short read sequencing (17). Using this process, in the 13 months from 1 May 2016 to 30 May 2017, *M. tuberculosis* was identified in 2,751 samples, sent from 2,252 patients (Fig. 1). Using these samples we derived and validated using an independent validation set (Fig.1) a strategy for investigating and controlling false positive variation between samples, which we here term *adaptive masking* (Fig. 2). The initial stages of adaptive masking involve estimating minor variant frequencies across the genome from mapped data, and determining whether these are related to the amount of non-Mycobacterial DNA present.

We identified 718 samples from 234 individuals from whom more than one positive sample had been obtained with 7 days of another. Of these, in six individuals samples were either reported as being of different lineages, as defined (30), differed in multiple drug resistances, or differed by >400 high quality SNVs. These observations we considered likely due to laboratory or sampling mix-ups, and samples from these patients were excluded. The other 700 samples were used as an independent validation set. From remaining samples, we identified the first sample from each of 2,018 individuals which were used to develop the adaptive masking strategy (Fig. 1).

Quantifying extraneous DNA and minor variant frequencies post mapping

We determined the proportion of non-Mycobacterial bacterial DNA in each sample using Kraken (23), mapped all reads to the H37Rv reference genome irrespective of Kraken results, and filtered the mapped data using stringent quality filters such that the expected error rate is expected to be less than 10^{-3} (see Methods). We defined 8,007 genomic regions in the reference genome; these regions comprise all canonical open reading frames and the genomic regions between them (Supplementary Data S1). We were unable to assess one 15nt region between two PPE family members (positions: 3380439 .. 3380453) as no high quality data mapped there in any sample.

In the other 8,006 regions, we observed that both minor variant frequencies and the relationship between minor variant frequency and the number of reads of non-Mycobacterial origin differed markedly by gene. For example, the *B55* and *esxW* genes had respectively very low and very high minor variant frequencies, independent of non-Mycobacterial DNA quantity. A small group of genes, of which the ribosomal component *rrs* is an example, showed low minor variant frequencies, except when non-Mycobacterial DNA was present (Figure 3A, B).

Estimating the impact of extraneous bacterial DNA

We modelled the relationship between minor variant counts and the number of non-Mycobacterial reads, divided into four approximately equal sized strata (Figure 3B), using Poisson models (Suppl. Data S2). Separate models were constructed for each region. Estimated minor variant frequencies in samples with <1% non-Mycobacterial bacterial reads had a median of $5x10^{-4}$ (Fig. 4A) across the 8006 genomic regions, which is compatible with the expected mapping error rate of $<10^{-3}$, given the filters applied.

The distribution of minor variant frequencies when less than 1% non-Mycobacterial bacterial DNA was present approximated a log-normal distribution with mean $\log(5x10^{-4})$ and standard deviation equal to the median absolute deviation (Fig. 4A, observed: black line; fitted, red line), but with a tail to the right. 208 regions (2.6% of the total 8006 regions), including esxW as well as other esx and PPE family members, had estimated minor variant frequencies > 2.1×10^{-3} when <1% non-Mycobacterial bacterial reads were present (Fig. 4A, Suppl. Data S2). This cutoff represents four median absolute deviations above the median; if the data were log-normally distributed, 24 samples would be expected with minor variant frequencies greater than this, vs. the 208 observed.

Overall, estimated minor variant counts rose as non-Mycobacterial DNA concentration rose, but for most regions the increase was small (Fig 4B): the median fold change in minor variant counts in the presence of >20% non-Mycobacterial DNA vs. <1% non-Mycobacterial DNA was 1.097 (i.e. a 9.7% increase, interquartile range 5.4% to 14.0%). 242 regions (3.0%) had statistically significant increases (Fig. 4B, 4C) of more than 50%. Most of these regions had the highest minor variant counts when >20% non-Mycobacterial DNA was present, although a small number had similar minor variant counts in the 5-20% range and the >20% range (Suppl. Fig. S2B).

Mutually exclusive regions with increased minor variant frequency

Comparing regions with increased minor variant rates with low (<1%) non-Mycobacterial bacterial DNA with those with increased minor variant rates with high (>20%) non-Mycobacterial bacterial DNA shows these regions to be mutually exclusive (Fig. 5). The former include PPE and *esx* family members, while the latter include ribosomal components (*rrl*, *rrs*, *rplB*, and *rps* genes) as well as other highly conserved bacterial genes (tRNA genes, *fusA1*, *infA*, *dnaK*, and others) (Fig. 5, Suppl. Data S2).

Examining Kraken read assignments in reads mapped to these highly conserved genes indicated that many reads mapping to these highly conserved regions cannot be unambiguously assigned to the *M. tuberculosis* taxon (Supp. Fig. 3) even when non-Mycobacterial bacterial DNA is not present, or is present in small amounts. For example, in the 422 samples for which <1% of *total* bacterial DNA is

248 non-Mycobacterial, only a small proportion (27.3%) of reads mapping with high quality to *rrs* are
249 assigned to the *Mycobacterium* genus by Kraken. However, since very little if any non-Mycobacterial
250 bacterial DNA is present, these reads are almost certainly derived from *M. tuberculosis rrs.* By
251 contrast, the corresponding figure for a gene with little homology with non-Mycobacterial genomes
252 (B55) is 97.4%. The corollary is that if one routinely removes reads which are not assigned by Kraken
253 to the genus of interest (in this case *Mycobacterium*), one will remove a very high proportion of the
254 reads corresponding to critical loci (including drug targets, such as rrs) even when no non-
255 Mycobacterial bacterial DNA, as occurs if one was sequencing pure cultures.

256 ## Adaptive masking reduces the reporting of biologically implausible inter-individual variation

257 A published strategy for excluding regions of high mapping variation within the *M. tuberculosis*
258 genome strategy masks (i.e. excludes from relatedness computations) 277,709 nt (6.3%) of the
259 genome (4). Excluding regions with high estimated minor variant counts with <1% non-
260 Mycobacterial DNA (zone A in Fig. 5B) adds an additional 1.1%. Excluding regions with increased
261 estimated minor variant counts only in the presence of >20% non-Mycobacterial bacterial DNA
262 (zones B-D) masks between 0.2% and 5.1% extra (Fig 5B,C). The masking of regions identified by
263 'adapting' to variation generated during the process forms the final part of the adaptive masking
264 process.

265 In a validation set comprising isolates taken with 7 days of each other from 234 individuals, using the
266 published strategy, 18/346 (5.2%) of pairs studied had ≥ 5 SNV variation, of which 10/346 were ≥
267 20SNV. On exclusion of region D, which comprises the four genes most influenced by non-
268 Mycobacterial DNA, all encoding ribosome associated products (the genes *rrl* and *rrs*, together with
269 the tRNA *metU*, and the highly conserved bacterial gene *tuf*), 0/346 pairs differed by >= 5 SNP ($p < 10^{-4}$ compared with the published method, Wilcoxon test on pairs). These genes were the only
271 genes with minor variant frequencies significantly affected by non-Mycobacterial DNA at the 1 to 5%
272 level (Figure 4B top panel; Supplementary Data Supplementary S2). Additional exclusion of genes in
273 regions B and C, mapping to which is less influenced by non-Mycobacterial DNA, had a limited
274 impact (Figure 6).

275 ## Impact of mapper

276 In the above work, we use the Stampy mapper(14) which forms part of the deployed TB
277 bioinformatics pipeline(17). To determine whether the choice of mapper was important, we
278 compared the results of mapping a 250 sample test set to the H37Rv reference genome using four
279 different software/parameter settings. We identified regions with any significantly increased
280 mixture frequencies when >20% non-Mycobacterial bacterial DNA was present relative to when <1%

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was present using a p-value of 0.05, adjusted by Bonferroni's method to 7.6×10^{-6}. The numbers of such regions detected with Stampy, bowtie, and bowtie2 with both stringent and more relaxed matching criteria were 2544, 25, 70, and 72 respectively (Supplementary Data S3). Increased in minor variants frequencies in *rrl* were detected by all techniques; estimated fold increases in minor variant frequencies associated with >20% non-Mycobacterial bacterial DNA were 37, 1.4, 1.5, and 1.5 respectively. Thus, although the mismapping observed identified occurs in all cases examined, it is much more prominent with Stampy than with the bowtie series of mappers.

DISCUSSION

Here, we describe an approach which we term adaptive masking. This involves monitoring the minor variant frequency across a bacterial genome to which sequencing reads have been mapped; as such, it measures an end product of next generation sequencing processes, taking into account the natural sequence variation in the samples studied, as well as the impact of DNA extraction and library construction technologies, and the performance of the mapping and filtering software used.

Using this approach, we defined a set of 'hard to map' genetic regions (zone A, Fig. 5) with increased minor variant frequencies irrespective of the amount of non-Mycobacterial bacterial DNA. Exclusion of these regions could be considered when assessing consensus *M. tuberculosis* sequences.

We also demonstrated a significant positive association between amounts of non-Mycobacterial bacterial DNA and minor variant frequencies in a subset of the mapped genome: significant increases of more than 1.5 fold were observed in 242/8006 regions examined, which together cover about 5% of the *M. tuberculosis* genome. Although the emphasis of this work is on relatedness between isolates, it is notable that included within the 242 regions are a series of genes encoding ribosomal components (*rrs*, *rrl*, *rpoB*, *rpsL*, *rpsA*) which correspond to major antituberculous drug resistance genes(31). Therefore, studies investigating resistance or heteroresistance using these loci should report estimates of the impact of the presence of non-Mycobacterial bacterial DNA on heteroresistance estimates. Such interference may be particularly marked when direct-from-sample short read sequencing is used(32), given the increased ratio of non-Mycobacterial : Mycobacterial DNA in the absence of selective Mycobacterial amplification using culture.

Among these 242 regions we identified four 'high variation' regions in which minor variant frequencies are very strongly influenced by non-Mycobacterial bacterial DNA quantities, with fold-increases in minor variant frequencies of >5 in the presence of >20% non-Mycobacterial bacterial DNA. Importantly, if non-Mycobacterial bacterial DNA concentrations are low (<1% of bacterial DNA present), as occurred in retrospective studies when *Mycobacteria* were subcultured on Lowenstein-Jensen slopes prior to sequencing, increased variation is not observed in these regions. The exclusion of the four 'high variation' regions from base calling by a clinically deployed *M. tuberculosis* pipeline markedly reduced reported variation between samples derived from the same patient in a short time period. In particular, prior to exclusion of the four high variation regions, in a test set derived from 234 individuals, 5.2% of intra-patient pairs examined differed by 5 SNV or more, with the majority of SNV differences observed in these pairs being >20. Multiple studies indicate this is biologically implausible (3, 5, 7), and after exclusion of the four 'high variation' regions comprising only 0.2% of the genome, no pairs had variation of 5 SNV or more. This suggests

that using standard 'masking' and DNA extraction from liquid media false positive variation is reported in a small number of sites in a non-Mycobacterial bacterial DNA dependent manner. Put alternatively, non-Mycobacterial bacterial DNA acts as an interfering substance (20) for relatedness measurements.

A potential limitation of this work is that this approach studies pre-specified regions of the genome, specifically coding regions and intergenic regions. This approach was chosen to avoid the challenges of analysing the 4.4 x 10^6 bases of the *M. tuberculosis* genome individually, with concomitant loss of statistical power. Therefore, as described the method may neither detect, nor allow selective masking of, small regions with high minor variant frequencies within genes. A second limitation is that we did not use of metagenomics classifiers, such as Kraken, to identify non-Mycobacterial 'interfering' DNA and eliminate it prior to mapping to the *M. tuberculosis* genome. We did not do this because we observed that for the highly conserved *rrs* genes, metagenomic classifiers cannot confidently assign reads to a genus level, likely because there is insufficient sequence variation within short read sequencing of *rrs* to allow this. Therefore, until longer read sequencing become available sequencing less conserved flanking genomic regions, a strategy of read removal based on metagenomics classification will eliminate a high proportion of *bona fide M. tuberculosis* derived reads in conserved genes, even in samples without any non-Mycobacterial bacterial DNA. Despite these limitations, the strategy chosen appears to be of use clinically, based on the reduction in likely false positive variation between serial samples from individuals.

The routine clinical of use of next generation sequencing is rapidly increasing (1, 4, 22). However, the reporting of microbial identity, resistotyping, and relatedness information requires complex, multistep processes whose outputs are dependent on specimen decolonisation, selective culture, DNA extraction, library construction, DNA sequencing, and bioinformatic analysis (4). Reagent batches, software versions, and equipment involved in the process are all subject to change over time. The adaptive masking approach we describe here represents a route to the quantitative monitoring the performance of the output of this pathway, identifying whether changes in process which may appear innocuous alter mapping and basecalling across the genome. We do not propose that the output from the adaptive masking process as demonstrated here with data generated by Public Health England and processed by particular bioinformatics tools should be used to generate a list of problematic genomic regions which can be universally applied. Rather, we envisage that the adaptive masking process will be performed as part of the acceptance of process change, and periodically as part of quality monitoring, under the exact conditions used in the clinical laboratory issuing NGS based results. A list of problematic positions to be ignored during

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358 relatedness calculations can then be fed into systems doing such calculations, such as
359 findNeighbour2 (27), which would apply such masking across all samples.

360 Generalizable to other organisms and mapping pipelines, the adaptive masking approach we
361 described here will have application in monitoring the performance of such processes quantitatively,
362 in interpreting estimates of possible heteroresistance, and in preventing the calling of false positive
363 variation in the context of clinically deployed genomics.

364

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FIGURE LEGENDS

Figure 1 Samples used, derivation and validation sets

A flow chart describing the samples used, and the selection of derivation and validation sets.

Figure 2 Bioinformatic Processes

A flow diagram illustrating the standard bioinformatics pipeline used, as well as the adaptive masking process used to generate masks. Gray circles denote links to a description of the process at https://github.com/davidhwyllie/adaptivemasking. FindNeighbour2 is an open-source server-based system for monitoring single nucleotide variation(27).

Figure 3 Minor variant frequency and non-Mycobacterial bacterial DNA quantities

The observed minor variant frequency for three regions of the *M. tuberculosis* genome (genes *B55*, *eswX*, and *rrs*) *vs*. the proportion of reads of non-Mycobacterial bacterial origin (as determined by Kraken) is shown for samples in the derivation set (n=2018). Panel (A) shows a dot plot, whereas in panel (B) the proportion of reads of non-Mycobacterial bacterial origin is stratified into strata with 1%, 5% and 20% boundaries. Numbers above each stratum refer to the number of samples with non-zero read depth in that region.

Figure 4 Modelling minor variant frequencies

For 8,006 genomic regions of the H37Rv reference genome, Poisson models were used to estimate the mean minor variant frequency. The estimated minor variant frequency when less than 1% non-Mycobacterial bacterial DNA is present (n=208, 2.6% regions) is shown in (A). The red line is a log normal distribution with μ = log(minor variant frequency with <1% non-Mycobacterial DNA) and σ = median absolute deviation(log(minor variant frequency with <1% non-Mycobacterial DNA)). In (B) the rate ratio estimates (i.e. the fold change associated with increases in non-Mycobacterial bacterial DNA quantifications) for each gene are shown. (C) shows the significance of a test comparing the log(rate ratio estimates) with zero, in the form of a Volcano plot. The dotted lines in (B) and (C) correspond to a 50% increase in rate ratio.

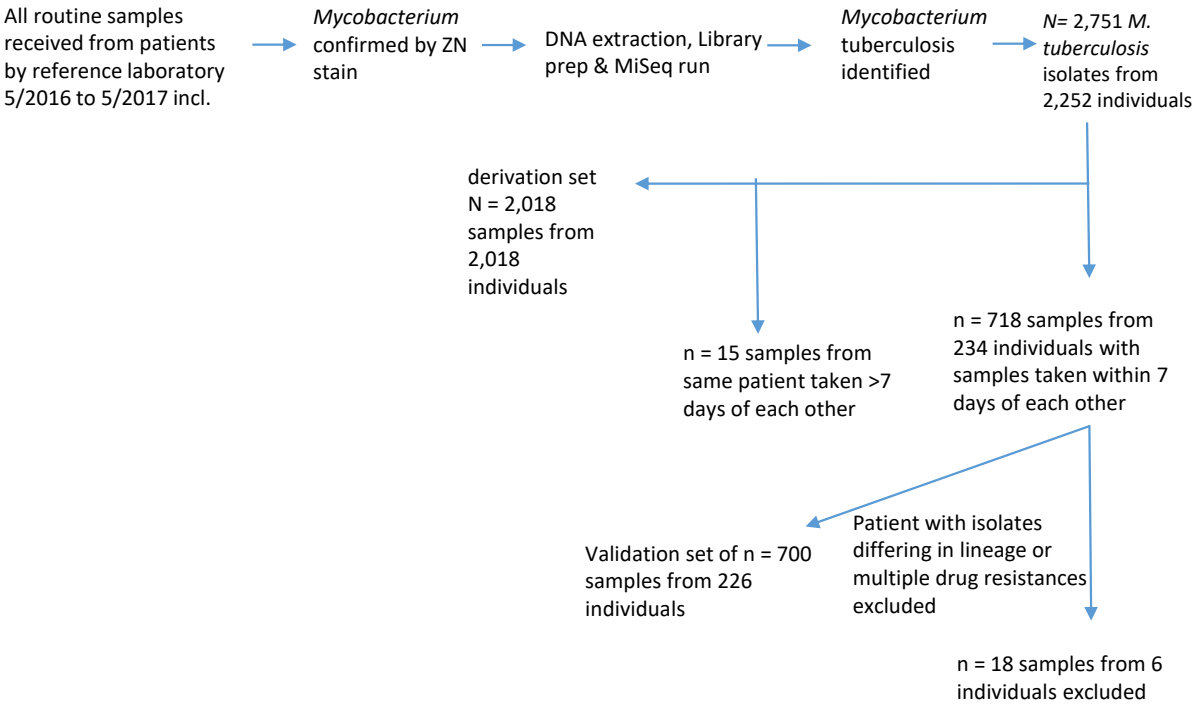
Figure 5 A distinct subset of genes are impacted by quantity of non-Mycobacterial DNA

Legend: (A) Fold change in minor variant frequency with > 20% non-Mycobacterial bacterial DNA present vs. <1% non-Mycobacterial bacterial DNA. Quadrant boundary markers correspond to (horizontal line) a 50% increase over <1% non-Mycobacterial bacterial DNA and (vertical line) a minor variant frequency of 2.1×10^{-3}. (B) Genes with elevated minor variant frequencies when non-Mycobacterial bacterial DNA is low (<1%) or high (>20%) fall into mutually exclusive sets. (C) the

number of bases represented by the deployed masking, vs. the deployed masking plus the genes in zones A, A+D, A+D+C, and A+D+C+B.

Figure 6 Impact of masking strategies on reported distances between closely related samples

SNV distances between pairs of *M. tuberculosis* genomes isolates from samples taken from the same individual within 7 days of each other were compared using different masking strategies. The top panel describes the published, deployed method of masking. In the panels below, genes in the zones shown in Figure 5B are additionally masked (i.e. ignored from pairwise comparisons).



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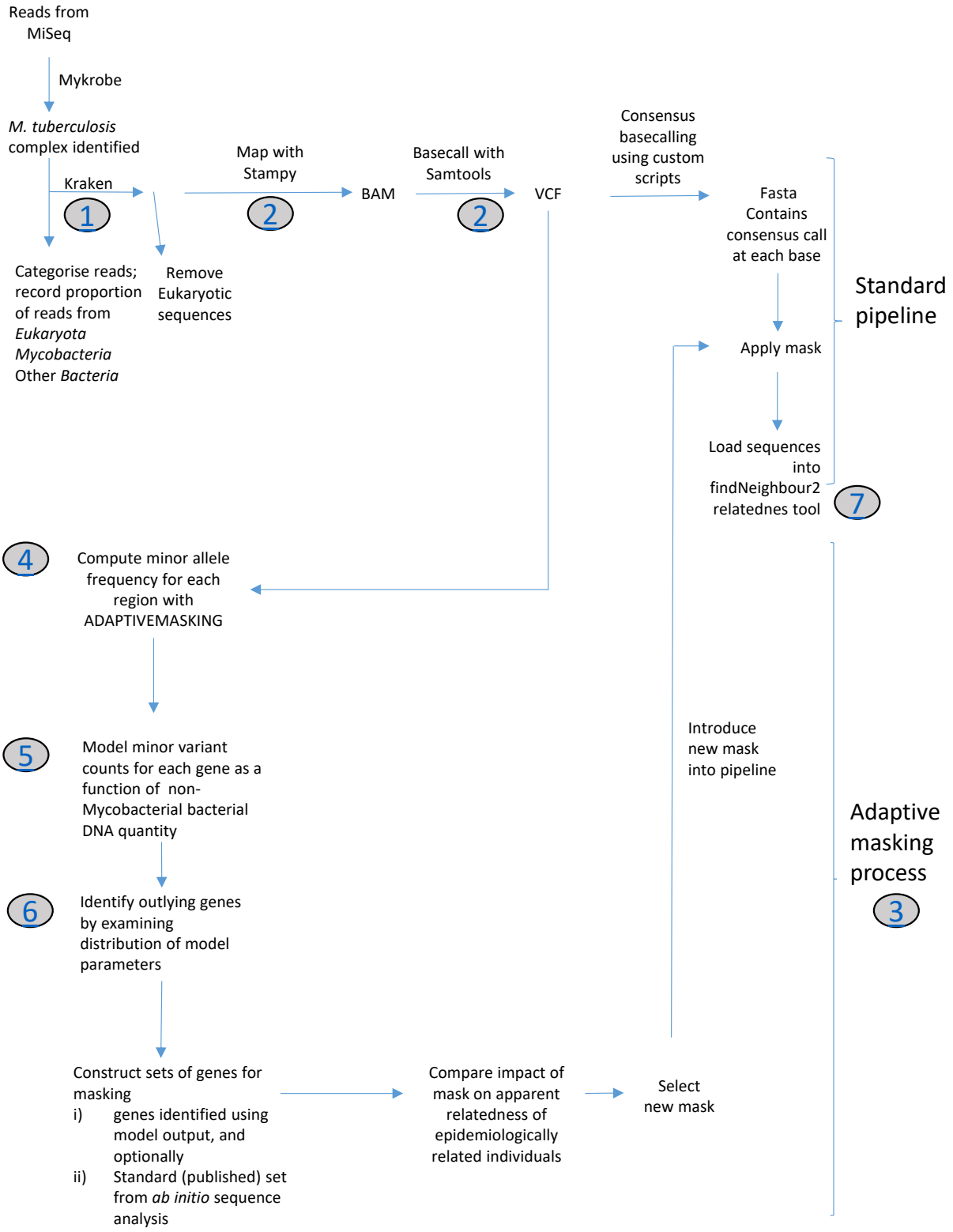

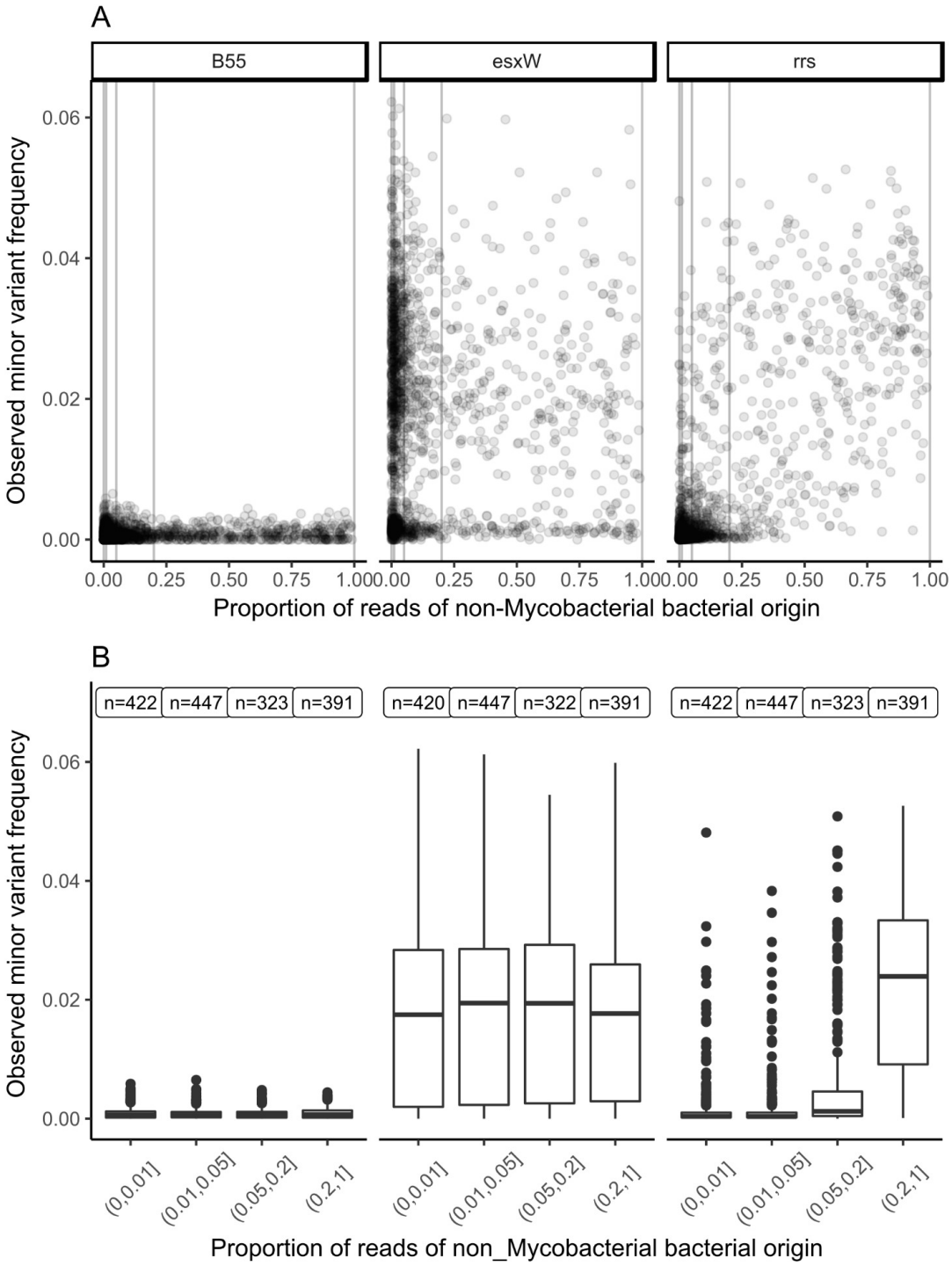

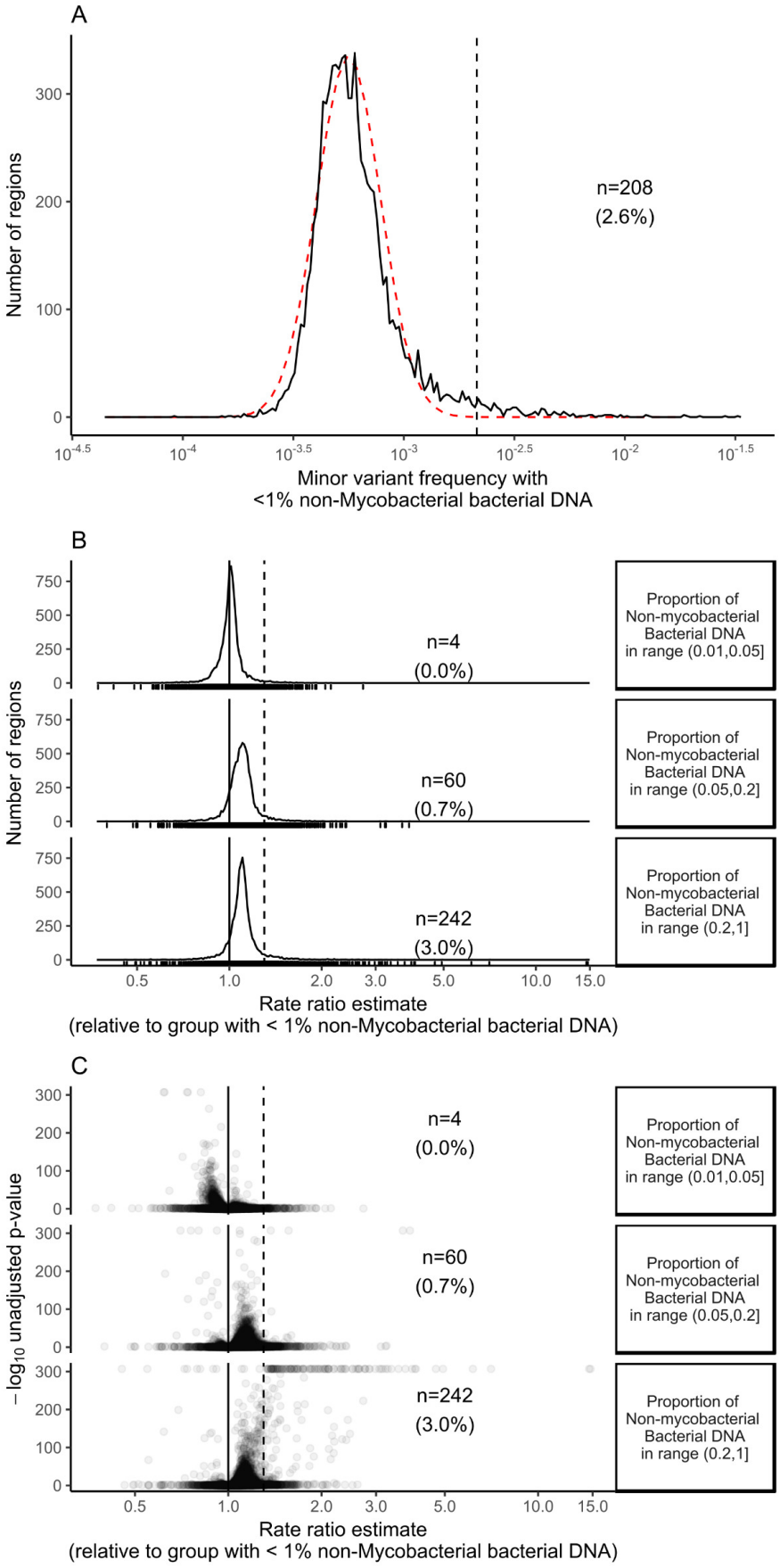

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